Androgen Receptor Binding Assay Update

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EDVMS Meeting

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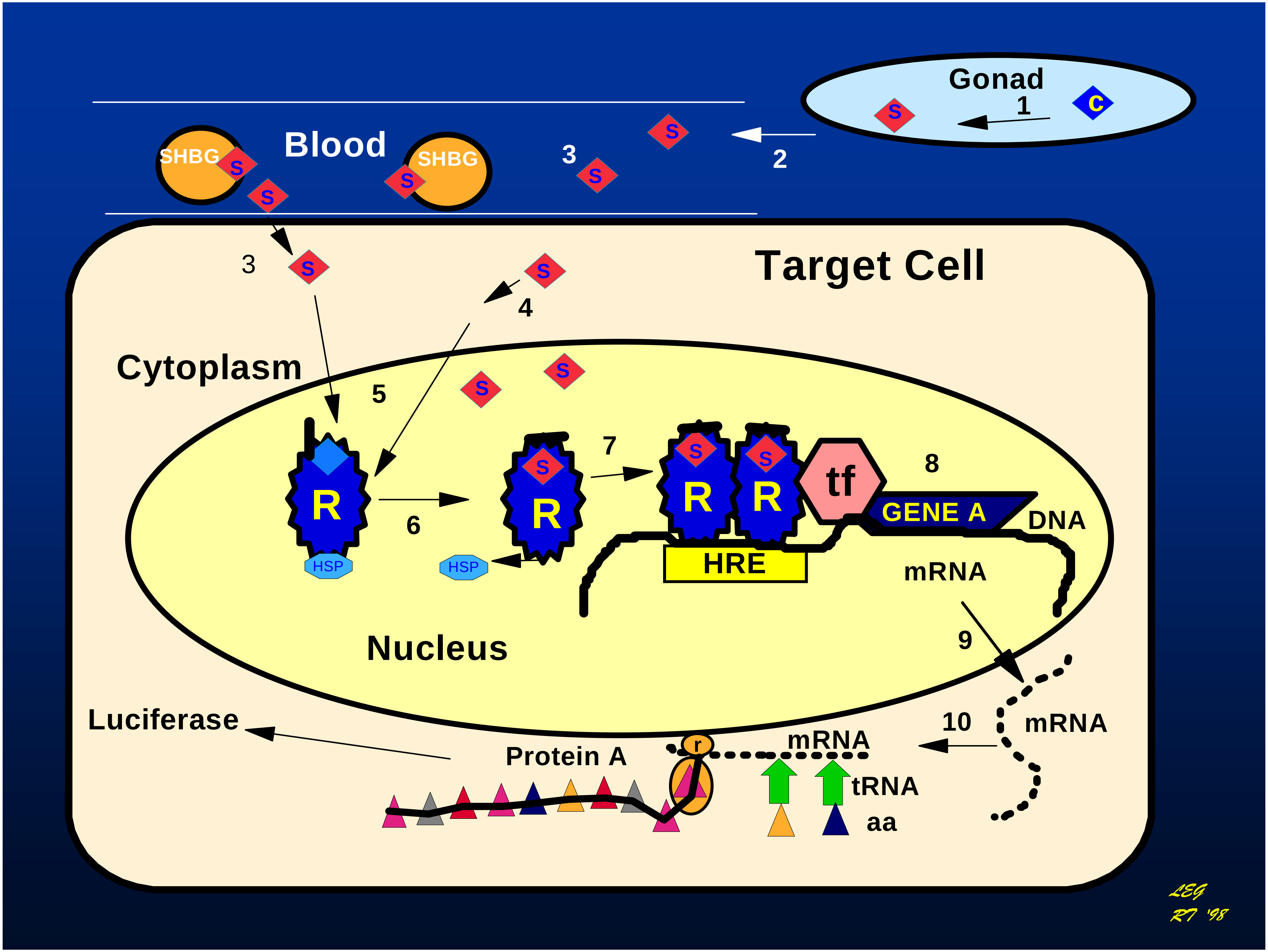
RESEARCH & DEVELOPMENT

Building a scientific foundation for sound environmental decisions

Overview

- General introduction to binding assays
- NICEATM/ICCVAM and Expert Panel
- Summary of work completed
  - Training and Protocol Refinement
  - Comparison of RPC and PV
  - Scatchard analyses
  - R1881 comparison
  - 16 chemicals
- Future Direction


Gonad
S
1
C
S
2
Blood
SHBG
S
SHBG
S
3
S
S
3
S
Target Cell
S
4
Cytoplasm
5
S
S
R
6
HSP
S
R
HSP
7
S
R
S
R
tf
8
GENE A
DNA
HRE
mRNA
9
Nucleus
Luciferase
10
mRNA
mRNA
Protein A
r
tRNA
aa
LEG
RT '98

Two basic types of receptor binding experiments

- **Saturation**

  Affinity of radioactive ligand for the receptor

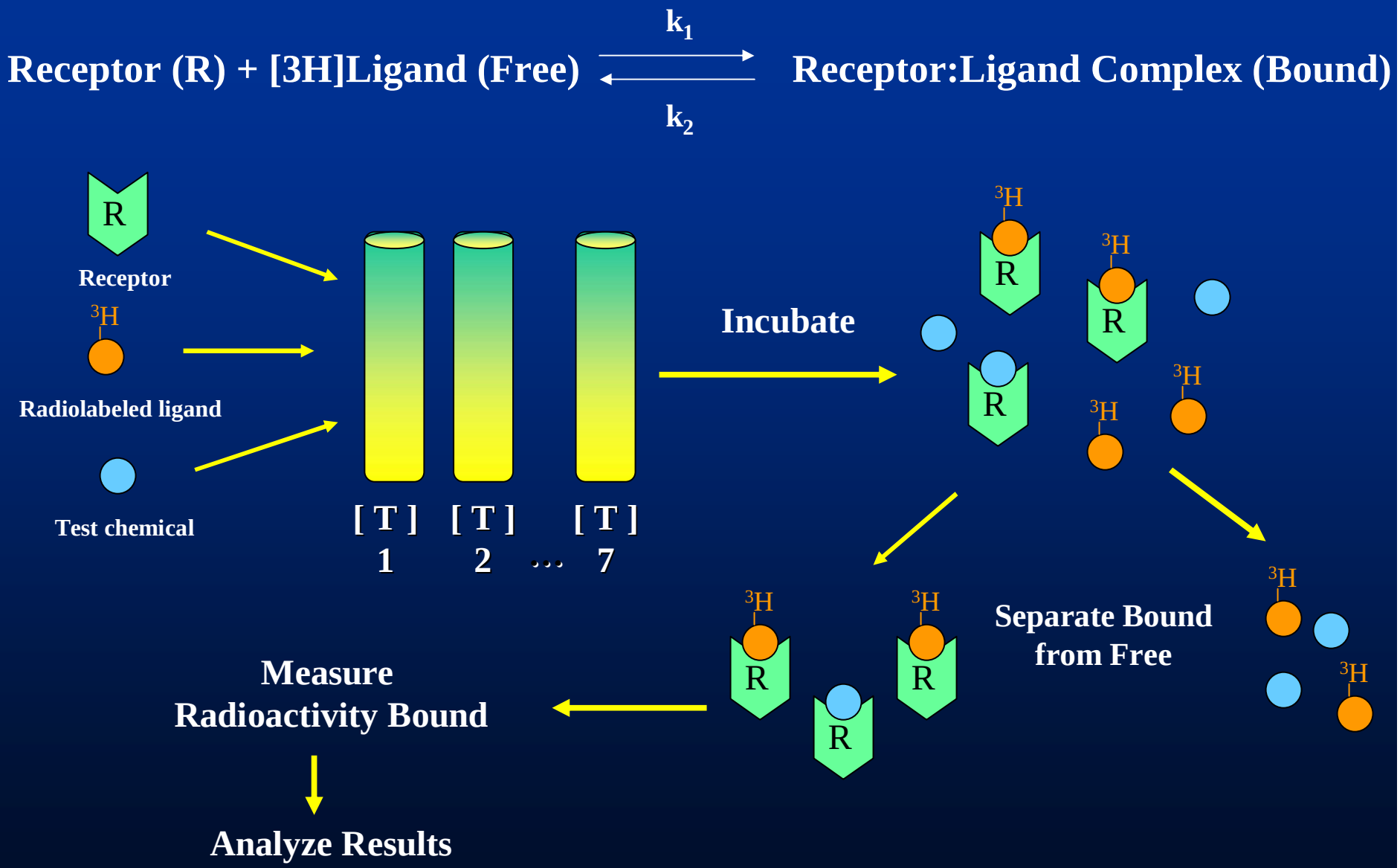
  - K_d - Affinity of radioligand
  - B_{max} - Binding sites

- **Competition**

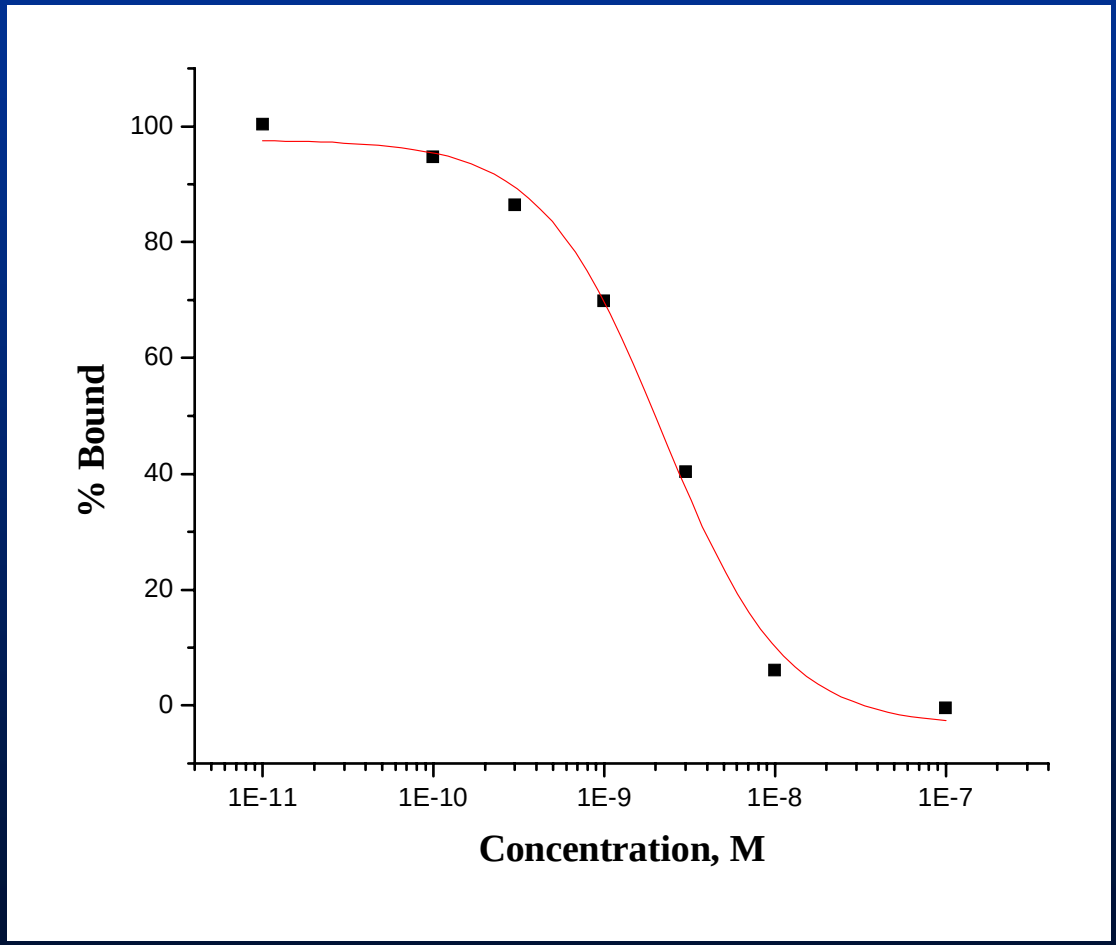
  Affinity of unlabeled ligand in competition with high affinity radioligand

  - IC50, RBA
  - K_i – affinity of unlabeled ligand

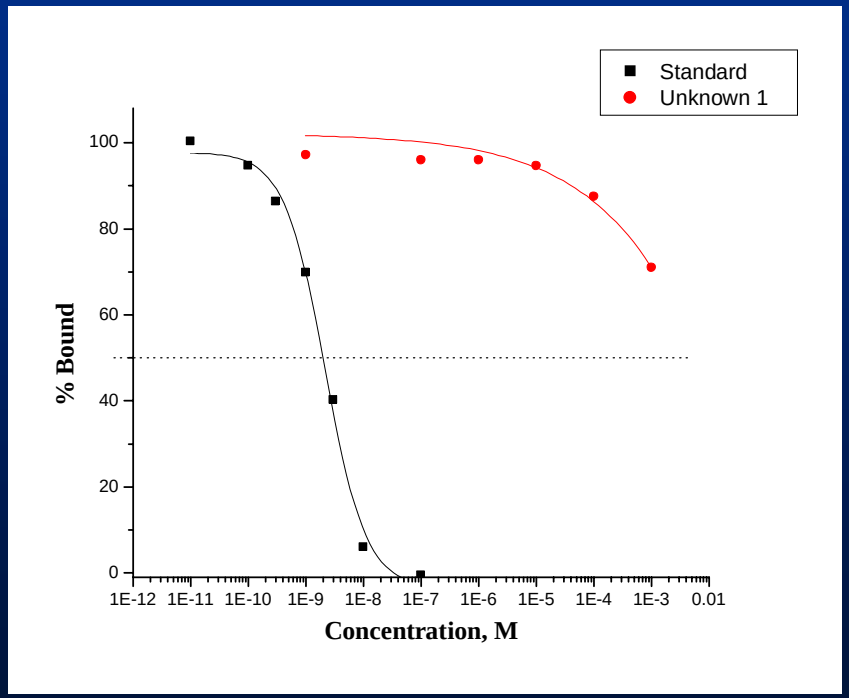
Basic Steps in Receptor Binding Assays

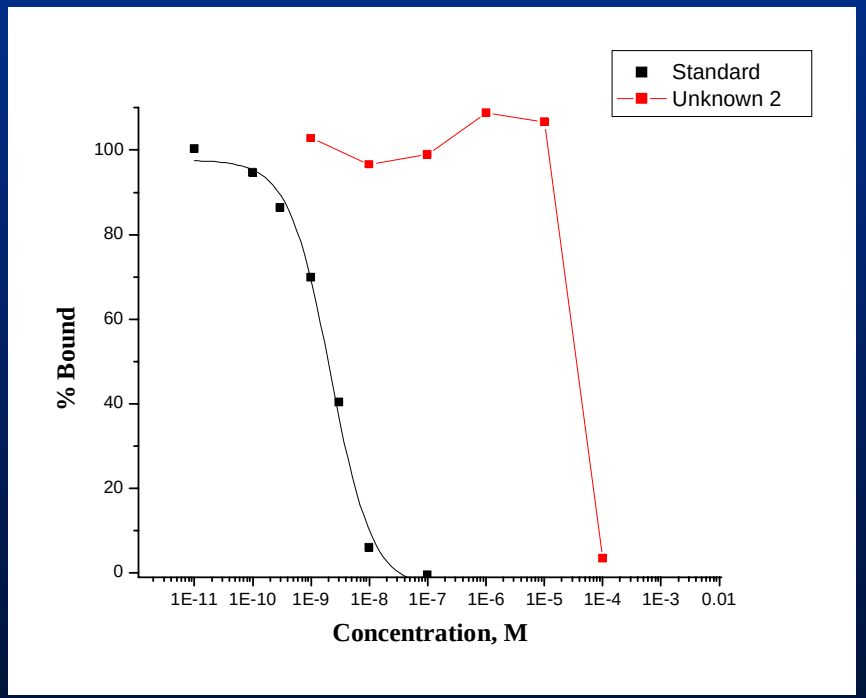


Competitive Binding Curve Quality Data

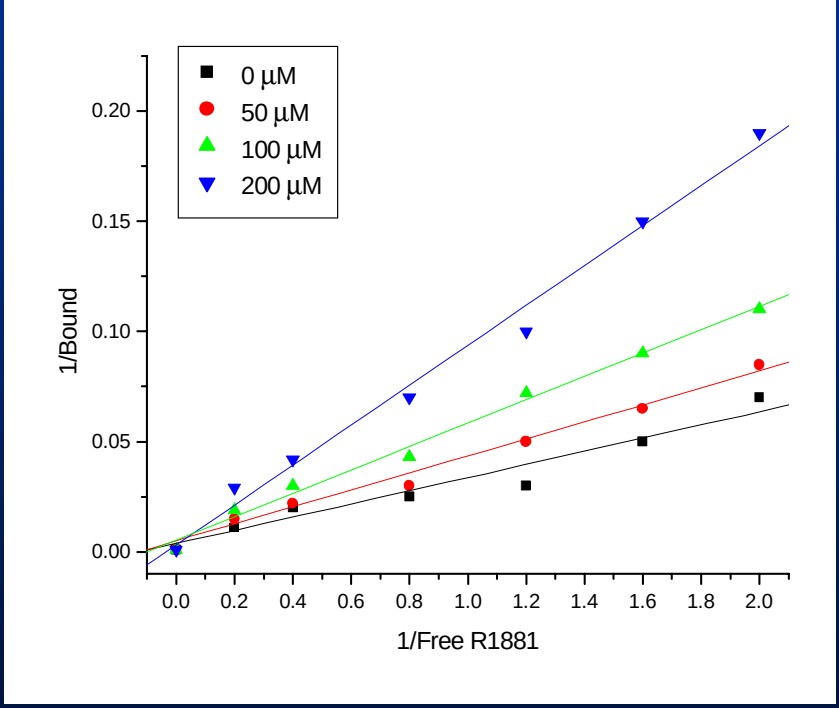


Example Binding Curves: Examine data carefully for problems

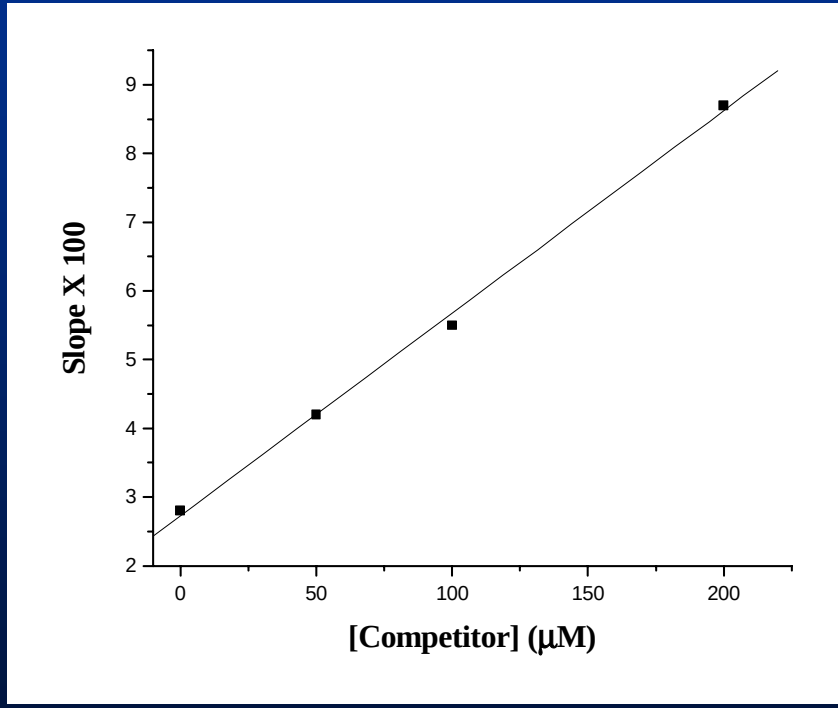




Experimental Determination of Competitive Inhibition and K_i



Double reciprocal plot



Slope replot

RESEARCH & DEVELOPMENT

Building a scientific foundation for sound environmental decisions

EDC Expert Panel Report

- Acknowledged the lack of a standardized in vitro AR binding assay protocol
- Identified need for establishing comparative performance criteria
- Agreed on minimum procedural standards
- Acknowledged that RPC is “Gold Standard” for comparison purposes
  - Most frequently used - Particularly useful as a reference
  - Has several disadvantages
- Recommended as high priority the development of an assay using purified, recombinant full-length AR
- Patent issues with hAR so an assay using an AR sequence from a species closely related to human may be necessary

RESEARCH & DEVELOPMENT

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  - 16 chemicals
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Comparison of RPC and PanVera Assays

2 Protocols

Rat Ventral Prostate Cytosol (RPC) - from EPA, RTD

PanVera - from NCTR

19 Chemicals over a range of potencies

Identified by number only

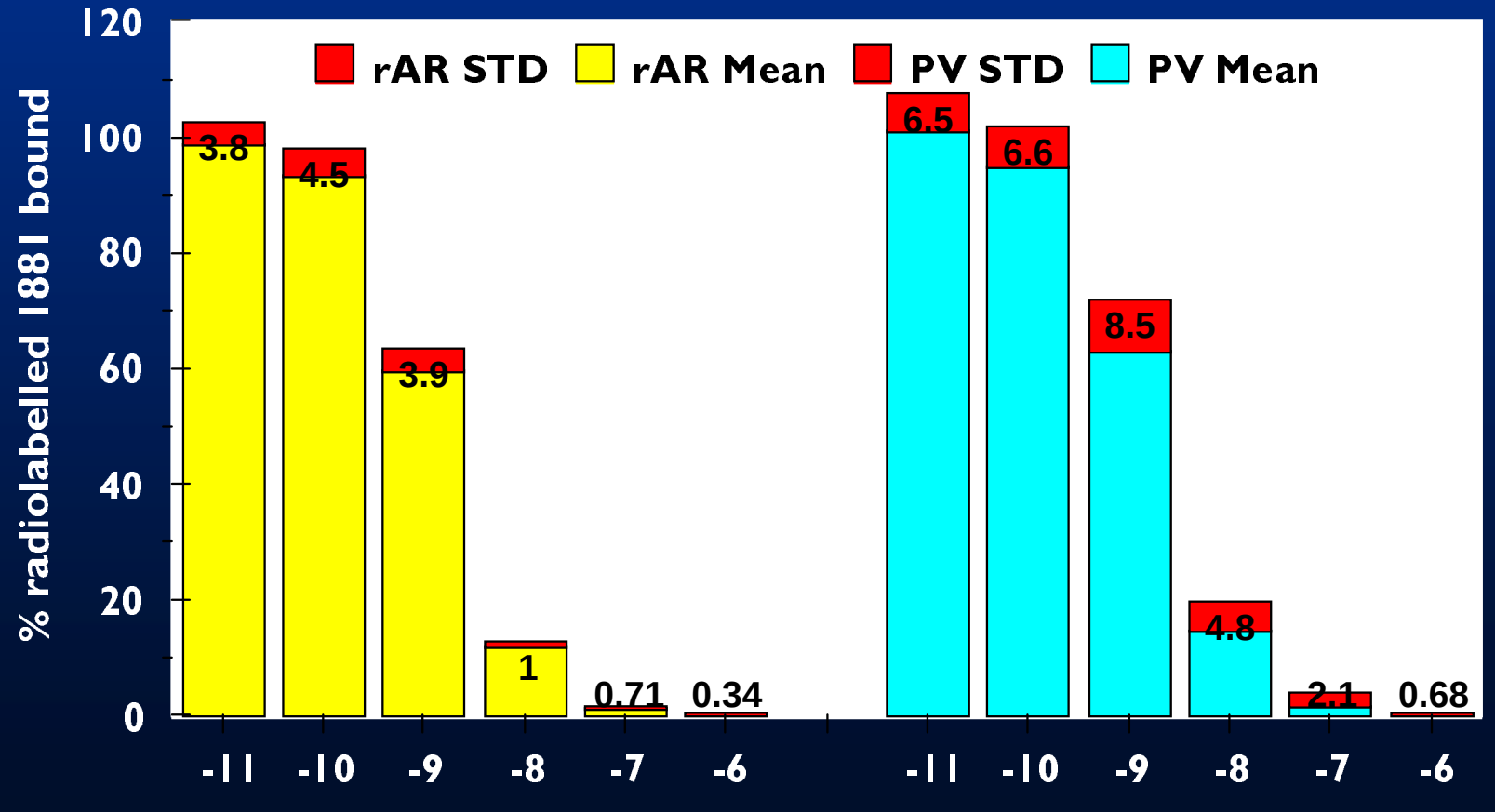
Design:

- 3 Technicians
- Each tech ran every chemical in both protocols
- 2 Duplicate tubes per run (3 runs in dup)
- Positives were repeated by all 3 techs (6 runs)

Test chemical concentrations as specified in each protocol

Comparison of RPC and PV binding assays for R1881.

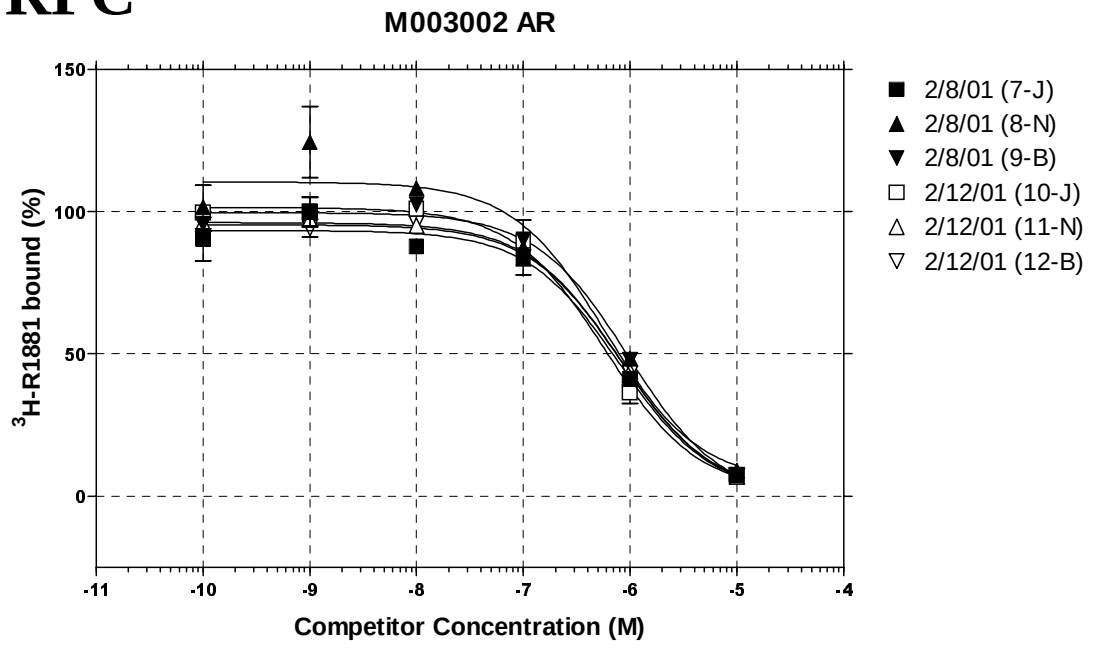
The interassay CV for the PV assay is 13% versus 6% for the RPC assay. Hence the PV assay is 2 fold more variable, which will require more replicates.



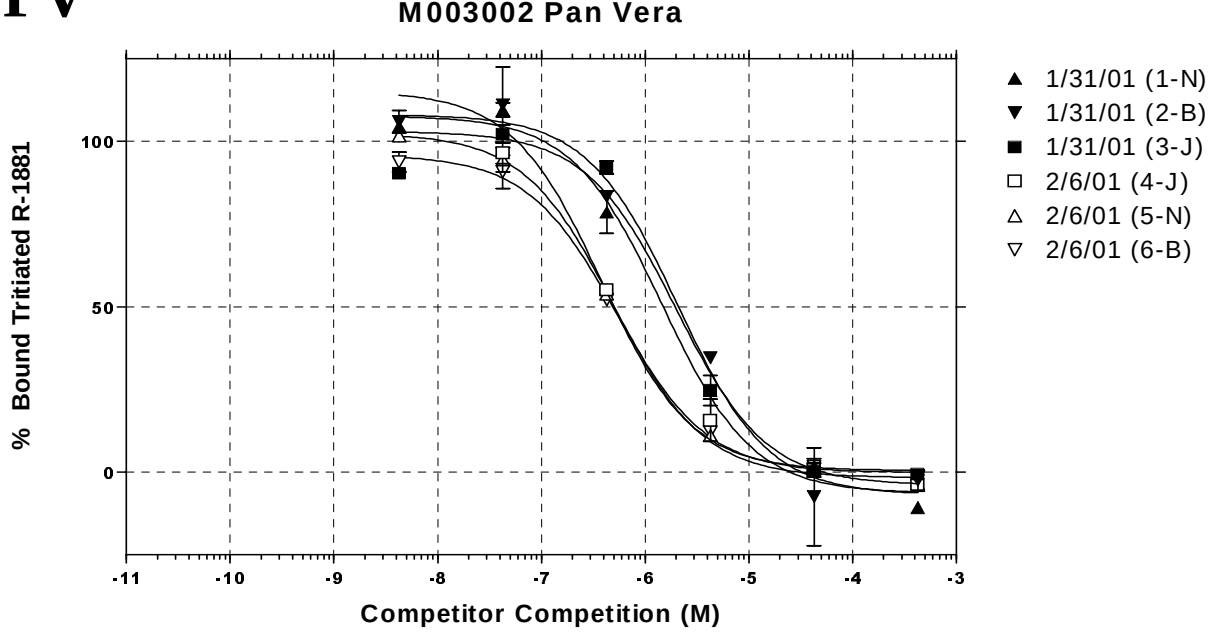
Binding Curves Example of Variability between runs

6 runs of same chemical in both protocols

RPC



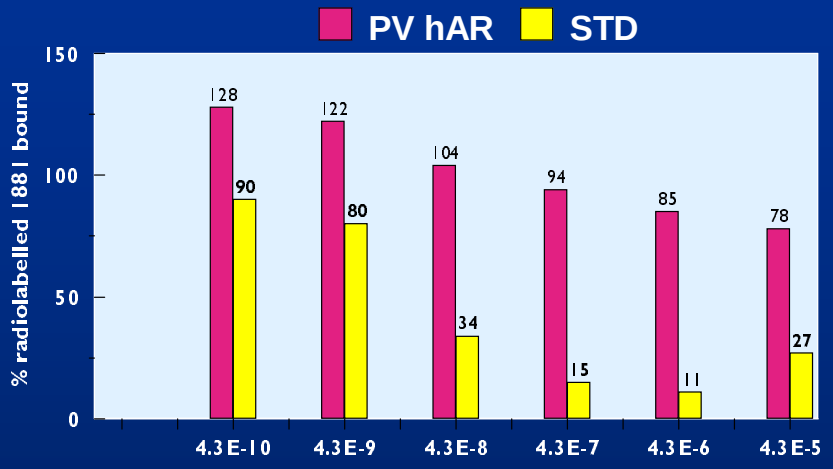
PV



Examples Illustrating Concerns with PV Assay

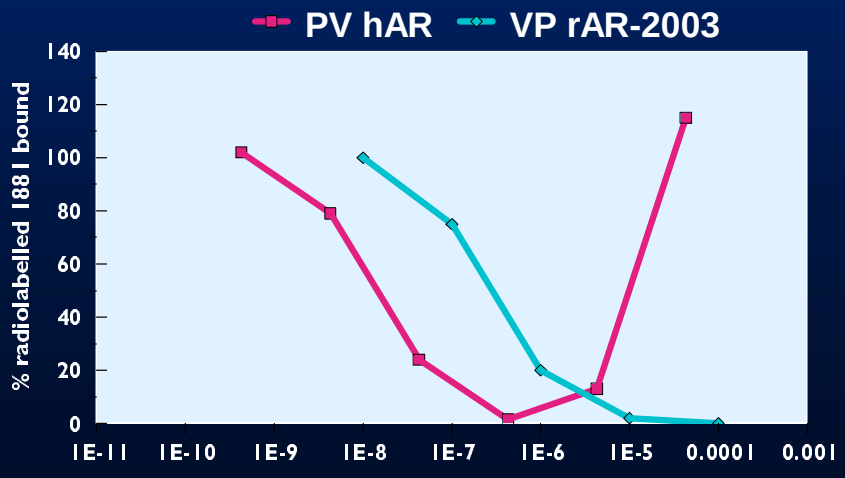
High Interassay Variability Binding Greater than 100%

PV Binding Assay for 3039 (DEHP)



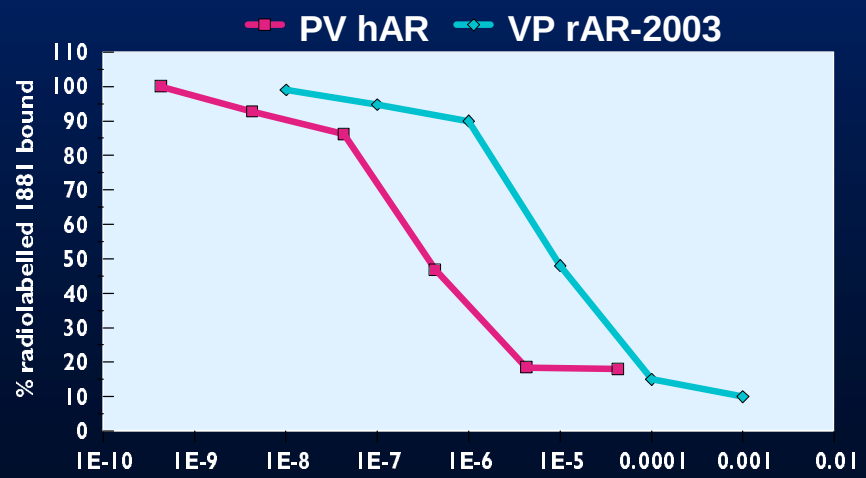
U-Shaped Curve

Comparison of RPC and PV for E2



Curve Shifted

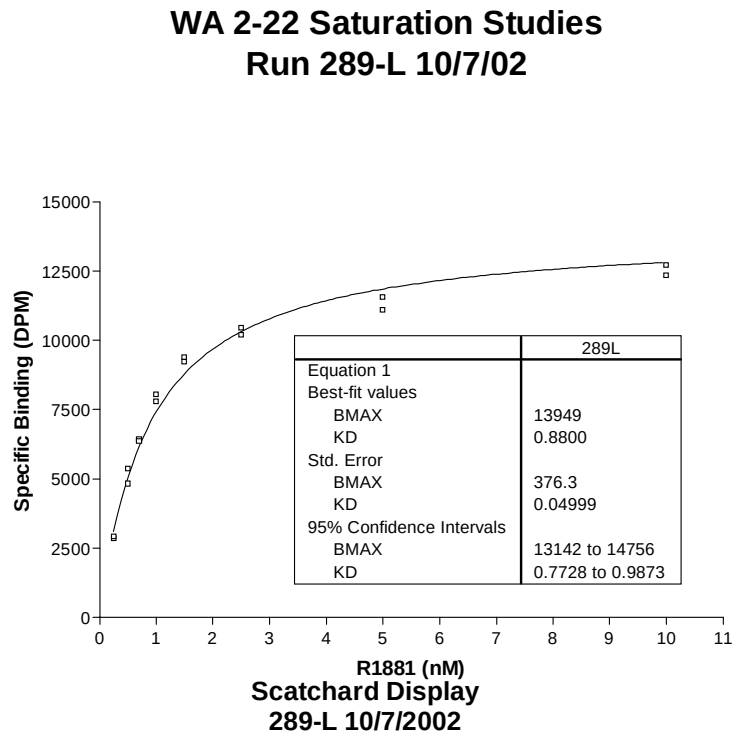
Comparison of RPC and PV for p,p'-DDE



Analysis of Assay Comparison

- High intra-assay variability in PV
  - 3.5% of duplicates rejected. Discrepancy of greater than 25%
- High inter-assay CV in PV assay
  - Twice the rejection rate of the RPC
- Several PV assays with extraordinarily high CVs
- Other Issues
  - Some U-Shaped binding curves in PV
  - Binding greater than 100% in some PV assays
- Different concentrations of unknowns used in RPC and PV assays complicates comparison of assays

Saturation Binding Acceptable



- Two technicians
- Two Runs per technician
- Duplicates per run
- Runs on two different days

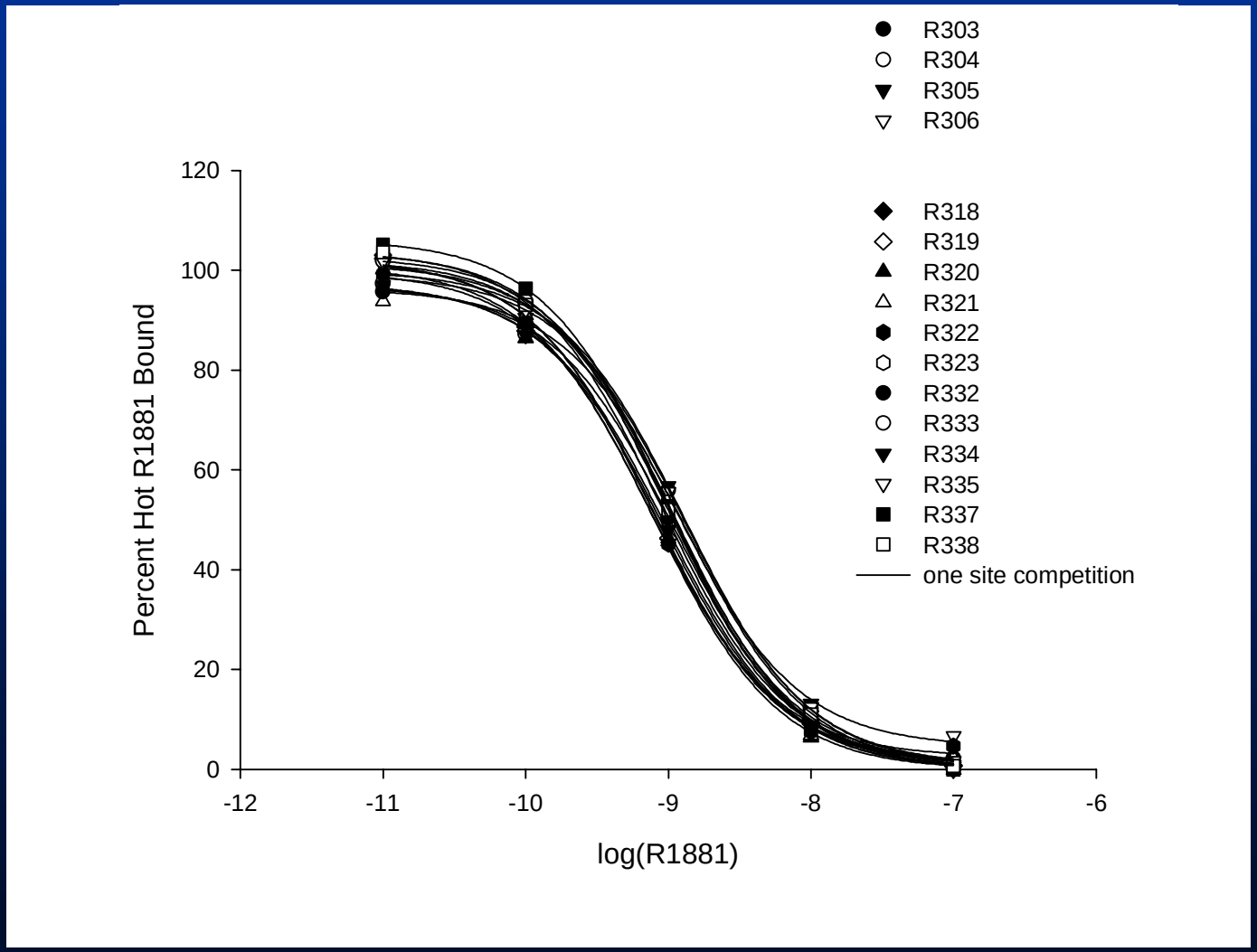
Run	Kd, nM	Bmax, fmol/mg
288J	0.9418	65.29
289L	0.880	64.75
290J	0.9615	66.0
291L	0.8710	64.59
Mean	0.914 +/- 0.04	65.16 +/- 0.64

Reference Chemical (R1881) Comparison

- 2 Technicians each ran twice with duplicates – 4 reps (Subtask 3.2)
- Repeated – 2 technicians; 6 runs each – 12 reps (Subtask 3.5) - Sixteen total replicates
- Analysis was a nested ANOVA with a 5 x 2 x 8 x 2 design (5 concentrations of R1881; 2 techs; 8 replicates per tech; 2 duplicate observations per replicate)

R1881 Binding

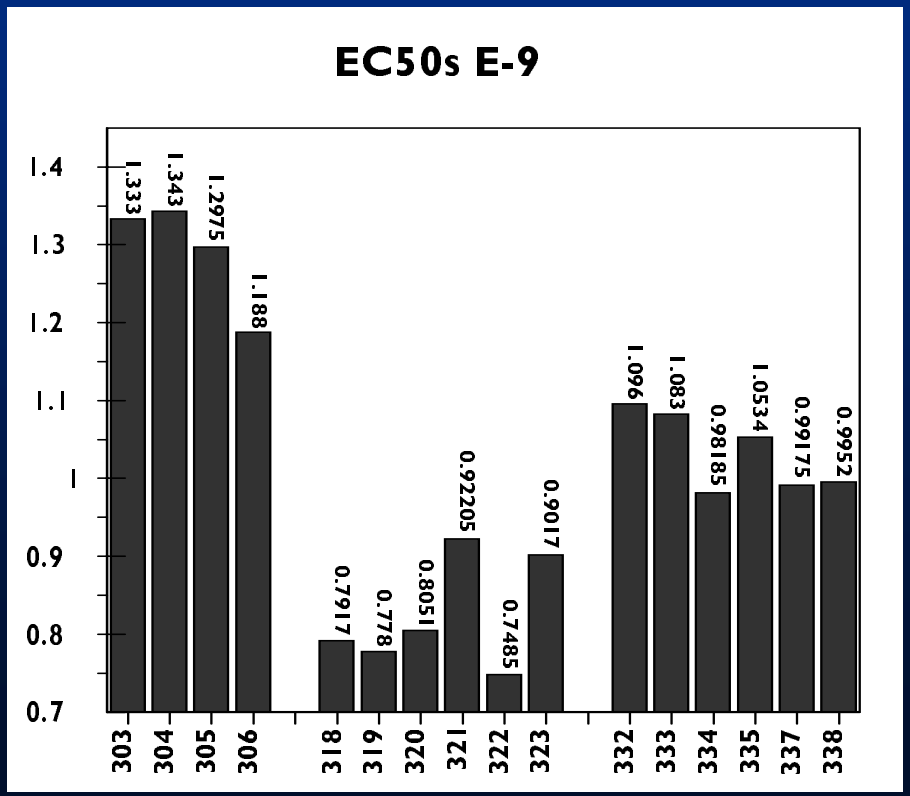
All runs converged and had R^2 values greater than 99%

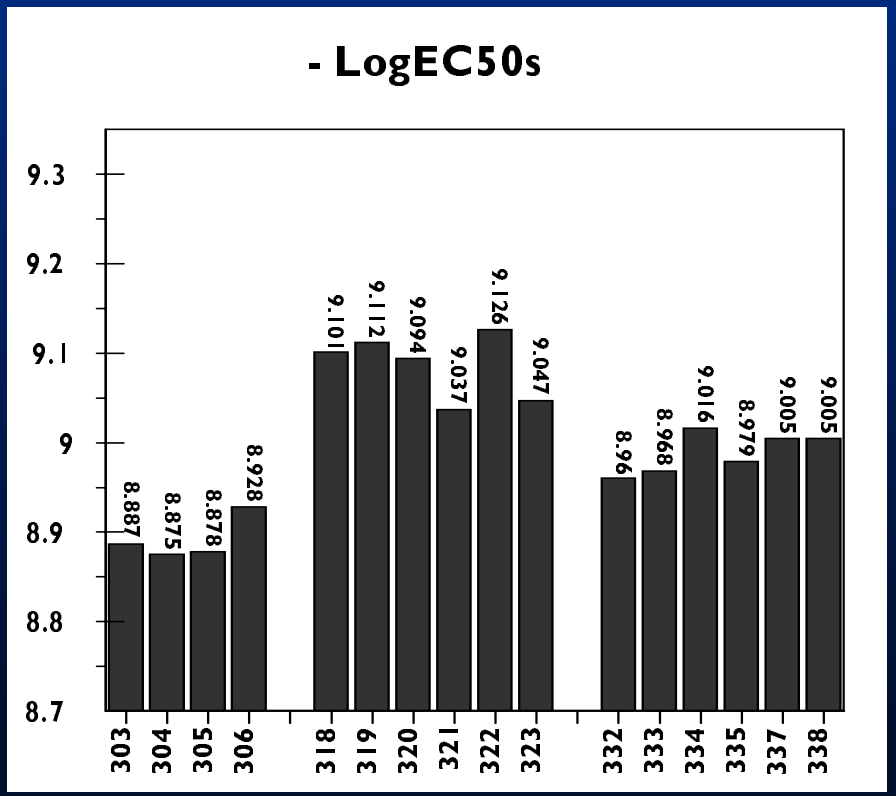


Sigma Plots's Ligand software

EC50 and log EC50 by Run

- Shows clustering of results over time
- CV of reps (8) within batch = 4.6%
- CV between batches = 22.5%
- Note similarity of reps between 2 technicians

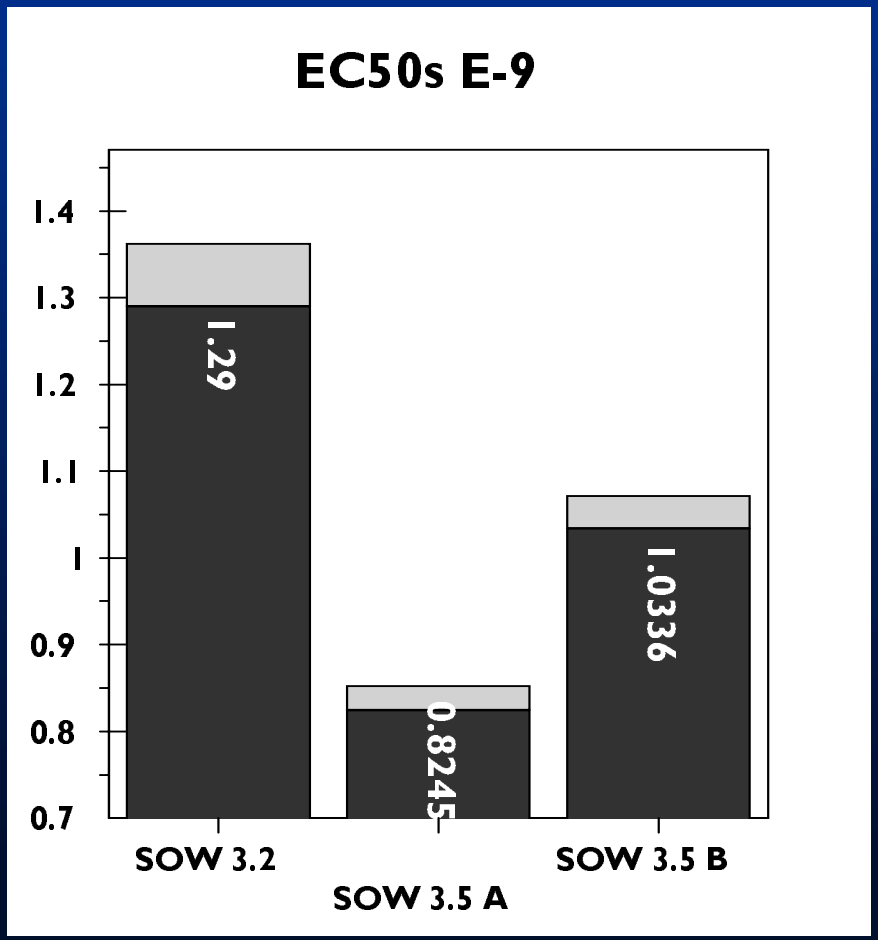


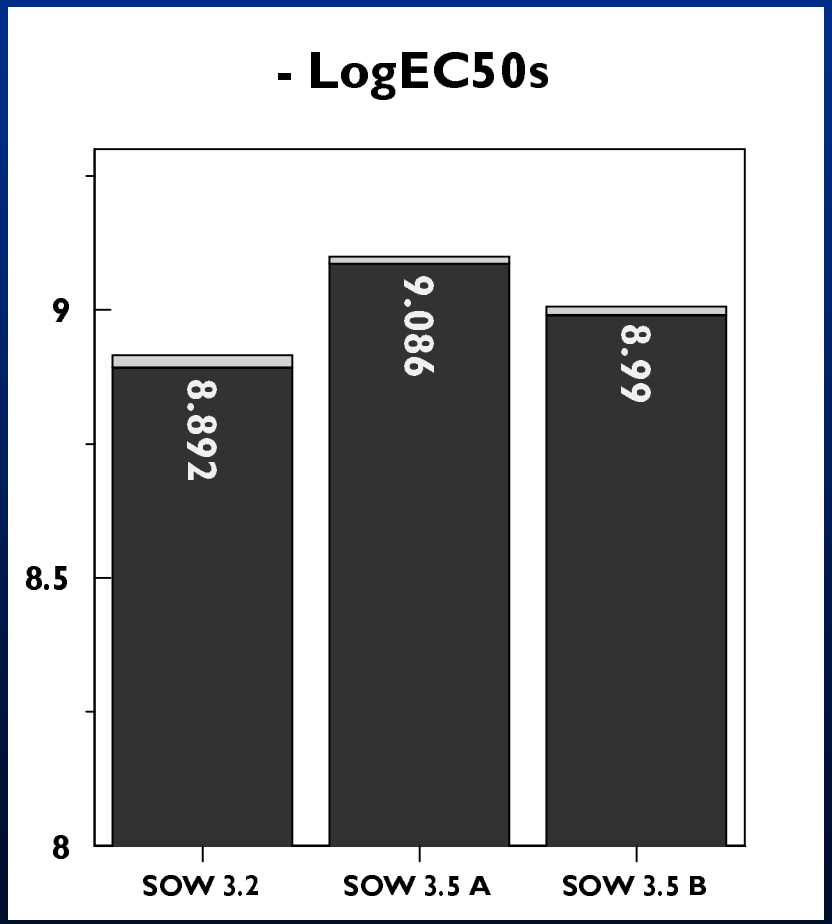


EC50 and log EC50 - Mean and SE
"Batch" Clustering of Results Over Time

All Three groups differ significantly from each other

CV between batches = 22.5%

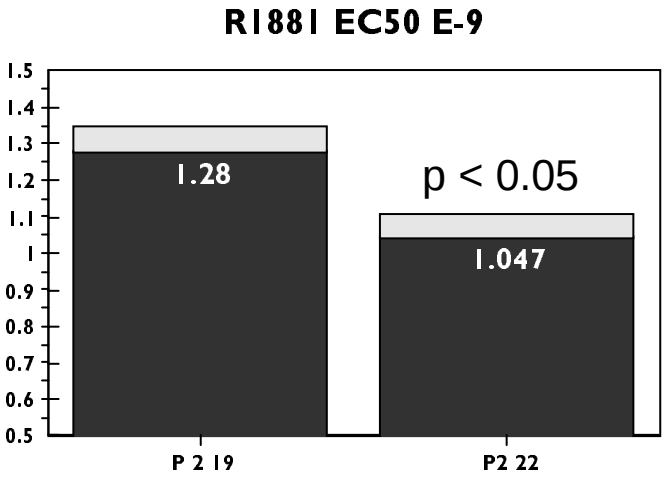


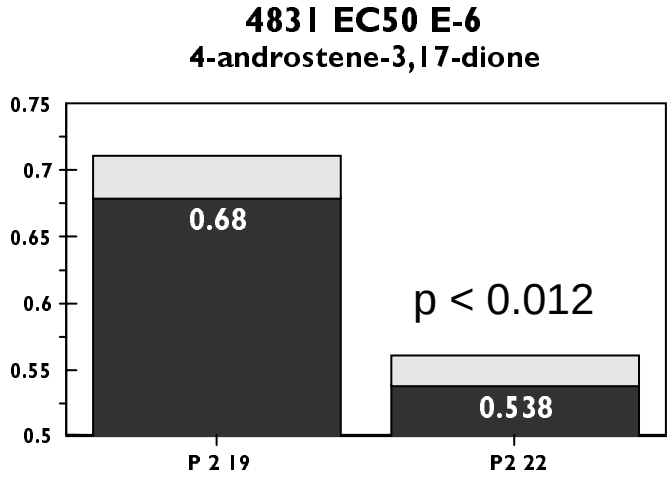


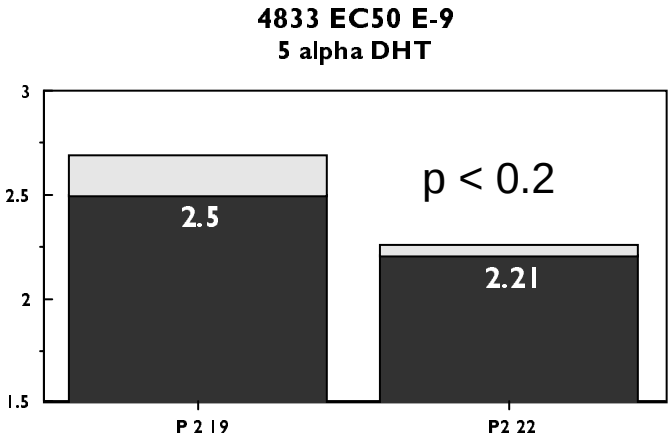
Summary and Conclusions
R1881 Comparison

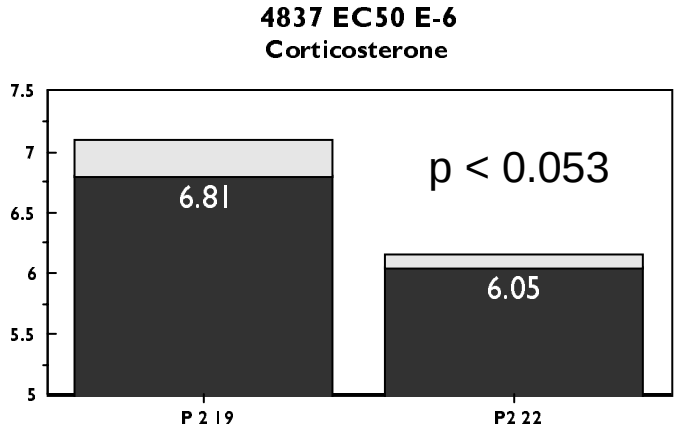
- Binding assay with R1881 was run 16 times in three "batches" by 2 technicians
- CV for duplicates – about 5%
- Interassay CV – about 22%
- Each run provided an excellent fit - R-squared values greater than 99%
- In the worst case, the IC50 values varied by 2 fold (0.7×10^{-9} to 1.3×10^{-9})
- Success

Task 3.3 AR Binding protocol comparison. Battelle concluded that there were only slight differences between the two protocols. However, we found that several of these were statistically significant. The "experiment" was unacceptable as designed, so such results should be ignored until the hypothesis is tested in a true side-by-side experiment.









Results of 16 Chemicals

- Original Report from Battelle classified
  - 14 Chemicals as Binders
  - 2 Chemicals as Non-Binders
- EPA Review reclassification
  - 10 Binders
  - 4 Equivocal
  - 2 Non-binders
- Equivocal binders - need additional experiments to define Ki
- Chemicals were each run 2-3 times but better experimental design needed before detailed statistical analysis

RESEARCH & DEVELOPMENT

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BINDERS

Linuron
Cyproterone Acetate
17β-Estradiol
P,p'-DDE
Medroxyprogesterone Acetate
Methyltrienolone
Testosterone
Progesterone
Dexamethasone
Spironolactone

EQUIVOCAL

4-tert- Octylphenol
Methoxychlor
Vinclozolin
Procymidone

NON-BINDERS

Atrazine
Di(2-ethylhexyl)phthalate (DEHP)

Recombinant Androgen Receptor

Expert Panel recommended as high priority the development of an assay using purified, recombinant full-length AR

- Patent issues with human AR
- Species closely related to human

Questions with truncated (chimeric) AR

Ongoing work at EPA, RTD

- Chimpanzee cDNA library obtained
- Screening for full length AR

Future Direction

- Supplement binding data of 16 chemicals with additional runs and conduct statistical analysis (intralaboratory)

- Work on recombinant system is being conducted but lags behind
  - desirable but 2-3 years for development and standardization
  - no commercial or non-commercial source available

- Move forward with RPC assay
  - standard data set
  - comparative performance criteria
  - interlaboratory study